RAPID SCREENING OF LACTIC ACID BACTERIA FOR RESTRICTION ENDONUCLEASE ACTIVITY

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Summary. A rapid microscale heparin Sepharose CL-6B affinity gel procedure was developed for detecting restriction endonuclease (RE-Nase) activity in a variety of lactic acid bacteria. RE-Nase-containing extracts free of DNA, RNA and nonspecific nuclease activity can be produced for forty or more strains daily and only 10-12 ml of each log phase culture was required for screening. RE-Nase activity was detected in several streptococci and lactobacilli. With appropriate modifications, this procedure should allow rapid detection of RE-Nase activity in other bacterial species.

Introduction

Genetic engineering of bacterial species has been widely used in basic research and for industrial applications. However, transformation of bacterial cells can be compromised by endogenous restriction endonuclease systems within particular strains. To improve transformation efficiency, it is desirable to determine which particular strains of a bacterial species have restriction endonuclease (RE-Nase) activity. A comprehensive survey of strains might lead to the discovery of novel RE-Nases or isoschizomers of known enzymes from more desirable sources. Our laboratory has previously isolated two RE-Nases (*Sth*117I and *Sth*134I) from the industrially important species, *Streptococcus thermophilus* (Solaiman and Somkuti, 1990; 1991). These RE-Nases were determined to be isoschizmers of *Eco*RII and *Hpa*II, respectively. In addition, evidence for RE-Nase activity was observed in other strains of this species. Consequently, a procedure was required to systematically determine RE-Nase activity in 41 strains of *S. thermophilus* in our laboratory. Although heparin Sepharose CL-6B has been shown to be a superior affinity gel for RE-Nase isolation (Bickel *et al.*, 1977), column chromatography with this affinity gel would be a tedious procedure for screening numerous strains of a bacterial species for RE-Nase activity. Therefore, we propose the use of Heparin Sepharose CL-6B in a microscale batch separation for rapid processing of cell extracts. Extracts that were free of DNA, RNA and contaminating nonspecific endonuclease were prepared from all 41 strains of *S. thermophilus* in

one working day. The procedure was also applied to other streptococci and lactic acid bacteria. The extracts can than be used in RE-Nase detection assays using various DNA substrates.

Materials and Methods

Bacterial strains and growth conditions

Streptococci (41 *S. thermophilus* strains, 19 *S. lactis* strains, 2 *S. faecalis strains* and one strain each of *S. faecium* and *S. agalactiae*) were grown and maintained in tryptone-yeast extract-lactose (TYL) broth at 37°C (Somkuti and Steinberg, 1979). Lactobacilli (11 *L. planarum* strains, 10 *L. bulgaricus* strains, 6 *L. acidophilus* strains, 3 *L. casei* strains and 3 *L. helveticus* strains) were grown and maintained in MRS broth (Difco Laboratories, Detroit, MI) at 37°C. Twelve milliliters of TYL broth and MRS broth were inoculated with an 0.5 ml aliquot from each of the overnight cultures, respectively. Media were supplemented with 20 mM DL-threonine to facilitate subsequent cell breakage (Chassy, 1976). The cultures were then grown for 4 h at 37°C.

Preparation of cell extracts

The cells were centrifuged (12,000 x g, 10 min at 4°C), washed twice with 10 mM Tris buffer (pH 7.6) and resuspended in 500 µl of the same buffer. Cells were disintegrated by incubation at 25°C for 1 h with 2 µg/ml mutanolysin (Sigma, St. Louis Mo.) and sonication (five 20 sec bursts), followed by centrifugation at 12,000 x g, 10 min at 4°C. The supernatant constituted the crude enzyme extract. Isolation of RE-Nase containing extracts with commercially available affinity gel Heparin Sepharose CL-6B, involved a microscale batch separation using 1.5 ml Eppendorf tubes. The crude extract (500 µl) was allowed to interact with 50 µl activated Heparin Sepharose CL-6B at 4°C for 0.5 h. The affinity gel was pelleted, decanted and washed 3 times with 500 µl 10 mM Tris-HCl (pH 7.6)-5 mM ß-mercaptoethanol (TB) buffer followed by two washes of 500 µl 150 mM NaCl in TB buffer. The washes removed DNA, RNA and nonspecific nucleases. The potentially RE-Nase positive extracts were eluted with 2x 50 µl of 400 mM NaCl in TB buffer. The RE-Nase assay consisted of testing 10 µl of the *S. thermophilus* extracts with 0.2 - 0.4 µg substrate (lambda bacteriophage, phi X-174 RF DNA or T7-coliphage), 100 µg BSA in 10 mM Tris-HCl (pH 7.6)-20 mM $MgCl_2$-10 mM ß-mercaptoethanol-50 mM KCl (TMBK) buffer in 20 µl final volume. The digestion was carried out for 1 h at 37°C and an additional hour at 45°C. RE-Nases from the majority of bacterial species other than *S. thermophilus* demonstrated an intolerance to concentrations of 200 mM NaCl in the reaction mixture. Thus, these extracts were dialyzed using a microscale method (Elble, 1992) prior to the RE-Nase assay. The digestions were stopped with 10 µl loading buffer (258 mM Tris pH 8.2-258 mM boric acid-7.5 mM EDTA) and then run on a 1.2 % horizontal agarose gel at 100V for 3 h. Gels were stained with ethidium bromide.

Results and Discussion

Supplementation of lactose broth with DL-threonine, the use of log phase cells and mutanolysin facilitated cell breakage of *S. thermophilus* strains. The protein yield in the crude extracts of the 41 strains varied from 0.60 to 1.75 mg/ml. Contaminating nonspecific nuclease, DNA and RNA were removed from the Heparin Sepharose CL-6B bound RE-Nase by successive washes with TB buffer and 150 mM NaCl in TB buffer. Ten of the 41 *S. thermophilus* strains demonstrated RE-Nase activity with lambda bacteriophage DNA, phi X-174 RF DNA and

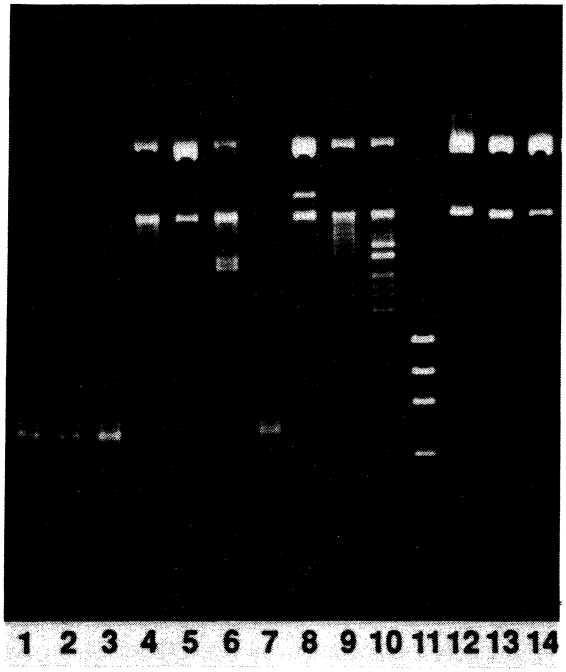

Figure 1. Detection of RE-Nase activity in heparin Sepharose-CL-6B derived enzyme extracts from *S.thermophilus* strains. The enzyme extracts were incubated with phi X-174 RF-DNA for 1h at 37°C and an additional 1 h at 45°C. The digests were ran on a 1.2% agarose gel and stained with ethidium bromide. Lanes 1-10: *S. thermophilus* strains; 11: *S. agalactiae*; 12: *S. faecalis; 13: S. faecium*; 14:ϕX174 RF-DNA

pBR322. The results of RE-Nase active and some nonactive strains tested with phi X-174 RF DNA are shown in Fig.1. The extracts from the two strains (ST117 and ST134), previously shown to have restriction endonucleases, demonstrated a restriction pattern with phi X-174 RF DNA that corresponded with results already reported (Solaiman and Somkuti, 1990;1991).

Potential RE-Nase containing extracts were prepared for strains of other streptococci species and lactic acid bacterial species with the procedures previously described. Optimum cell breakage of other streptococci required similar conditions to that of *S. thermophilus* and yielded similar protein concentrations. In contrast, supplementation of MRS broth with DL-threonine had no affect on cell breakage of lactobacilli. Incubation of lactobacilli with mutanolysin resulted in only a slight increase (1.33-fold) in protein content of cell extracts as compared to cells sonicated without mutanolysin treatment. Restriction endonuclease activity was tested for extracts from other streptococci species and lactic acid bacteria using lambda bacteriophage and phi X-174 RF DNA (data not shown). RE-Nase activity was observed in *S. agalactiae, S. faecalis* and *S. faecium* and a few *S. lactis* strains. In addition, a few strains of *L. bulgaricus* have weak RE-Nase activity. To date, observation of restriction endonuclease activity in *Lactobacillus*

species has been limited to one restriction enzyme in *L. casei* (Chassy, 1976) and a restriction-modification system in *L. helveticus* (Roberts and Macelis, 1991). In addition to *Sth*117I and *Sth*134I isolated in our laboratory (Solaiman and Somkuti, 1990;1991), several other strains of *S. thermophilus* have been shown to carry an RE-Nase that is an isoschizomer of *Kpn*I (Roberts, 1990). RE-Nases have also been isolated from various strains of *S. faecalis* and *S. agalactiae* (Fitzgerald *et al.*, 1982). An Re-Nase was isolated from an *S. lactis* strain (Reyes-Gavilan *et al.*, 1990) and restriction modification systems have been observed in several strains of *S. lactis* (Chopin *et al.*, 1984; Davis *et al.*, 1993).

The use of the microscale procedure for the detection of RE-Nase activity in strains of other bacterial species might require certain adjustments as determined by past observations of optimum conditions for cell breakage and RE-Nase activity in the same or related species. Some restriction endonucleases might require a higher concentration of NaCl for elution from the affinity gel. Furthermore, the TMB buffer used in the RE-Nase assay can be replaced with the universal buffer (McClellan *et al.*, 1988) or a more appropriate buffer for the particular bacterial species studied. With appropriate modifications, this procedure allows for the rapid determination of RE-Nase activity in a variety of bacterial species that might hinder or reduce the efficiency of genetic transformation.

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